Warfarin exposure and calcification of the arterial system in the rat

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Summary. There is evidence from knock-out mice that the extrahepatic vitamin K-dependent protein, matrix gla protein, is necessary to prevent arterial calcification. The aim of this study was to determine if a warfarin treatment regimen in rats, designed to cause extra-hepatic vitamin K deficiency, would also cause arterial calcification. Sprague-Dawley rats were treated from birth for 5–12 weeks with daily doses of warfarin and concurrent vitamin K1. This treatment causes an extrahepatic vitamin K deficiency without affecting the vitamin K-dependent blood clotting factors. At the end of treatment the rats were killed and the vascular system was examined for evidence of calcification. All treated animals showed extensive arterial calcification. The cerebral arteries and the veins and capillaries did not appear to be affected. It is likely that humans on long-term warfarin treatment have extrahepatic vitamin K deficiency and hence they are potentially at increased risk of developing arterial calcification.

Keywords: warfarin, matrix gla protein, calcification, vitamin K-dependent

Warfarin therapy creates an effective vitamin K deficiency. This results in decreased activity of the vitamin K-dependent blood coagulation factors, II, VII, IX and X and proteins C and S. These factors need vitamin K as a cofactor for the postribosomal carboxylation of certain glutamic acid residues to Ca^{++} binding γ-carboxyglutamate (gla) residues. In the non- or partially carboxylated state the blood clotting factors have little activity (Esnouf & Prowse 1977; Friedman *et al.* 1977).
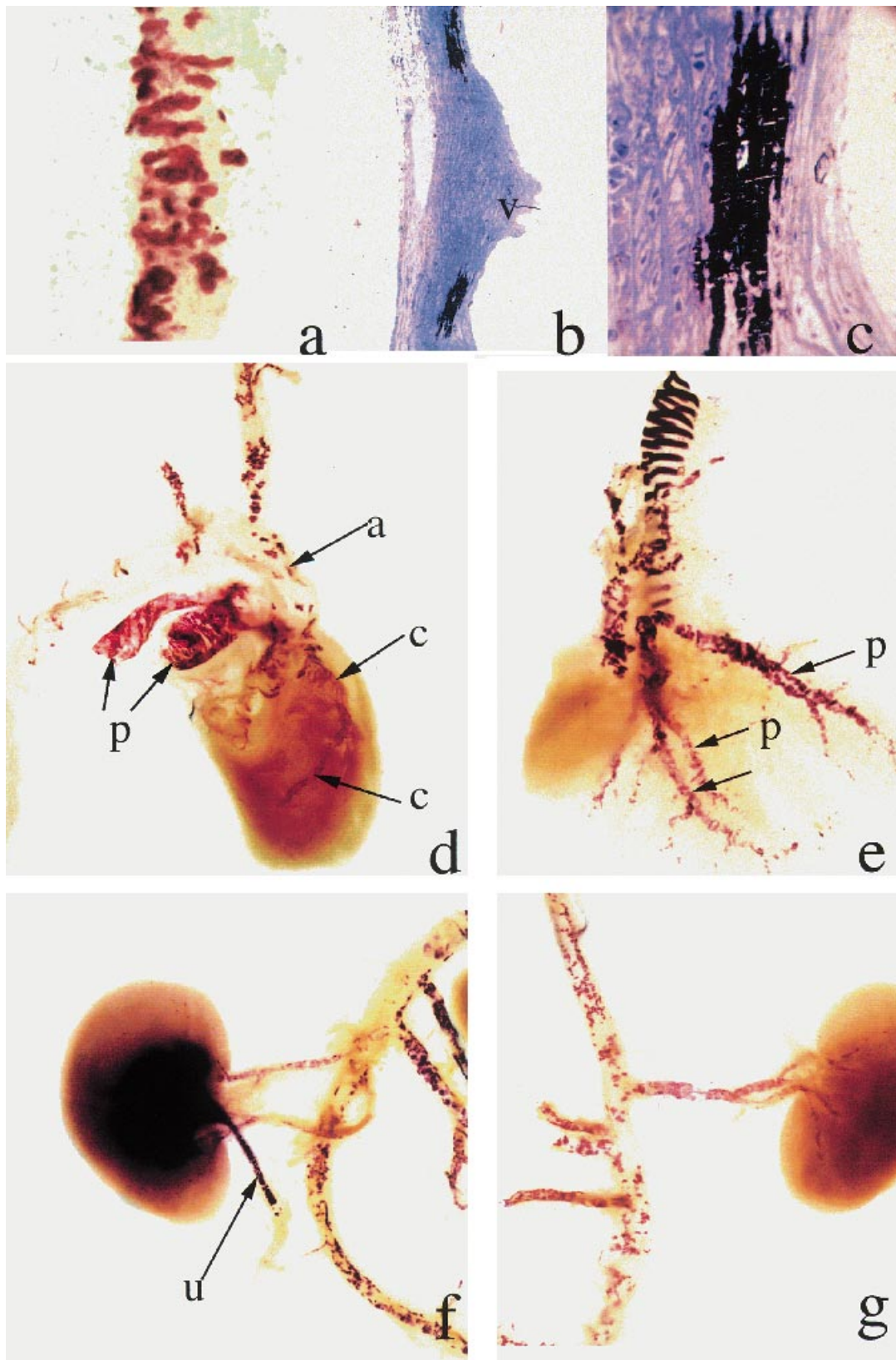
As well as the vitamin K-dependent blood clotting proteins produced by the liver there are a number of extrahepatic vitamin K-dependent proteins including matrix gla protein (MGP), osteocalcin, nephrocalcin, plaque gla protein, Gas 6 and proline rich Gla proteins (PRGP) 1 and 2 (Ferland 1998). Reduced carboxylation

of these proteins is likely to be an unavoidable side-effect of warfarin therapy.

The functions of these various extrahepatic proteins are largely unknown but recent studies with knockout mice give some indications. The MGP knockout mice developed severe calcification of the arterial system suggesting that MGP normally prevents this process (Luo *et al.* 1997). Osteocalcin seems to play a role as a negative regulator of bone formation with osteocalcin knockout mice having a higher bone mass but no other obvious abnormalities (Ducy *et al.* 1996).

It seemed likely to us that the arterial calcification seen in the MGP knockout mice might also be caused by long-term warfarin treatment. In view of our interest in the role of vitamin K deficiency in pre- and post-natal development we examined rats that had been treated for variable periods with both warfarin and vitamin K1 concurrently. This regimen, first described by Price & Williamson (1981), permits the treated rat to produce normal vitamin

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K-dependent blood clotting factors while creating an extrahepatic vitamin K deficiency. Hence the rats should have uncarboxylated or partially carboxylated MGP and osteocalcin while their blood clotting factors should be normal. Histological examination of these rats revealed that they had developed extensive calcification throughout their arterial system after only 5-weeks treatment. These results suggest that similar pathology may develop in humans receiving long-term warfarin treatment.

Materials and methods

Sprague-Dawley rats obtained from Gore Hill Animal House, University of Sydney were given rat food and tap water *ad libitum* and maintained under controlled conditions. These experiments complied with the National Health and Medical Research Council guidelines on the care and use of animals for scientific purposes and were approved by the Animal Ethical Committee of the University of Sydney.

The animals presented in this report were part of a study looking at nasal septum calcification as a result of warfarin-induced extrahepatic vitamin K deficiency. Ten rats were treated daily with a subcutaneous injection of sodium warfarin (100 mg/kg) (100 mg/ml in distilled water) (supplied and assayed by Boots Company, North Rocks, Sydney) and 10 mg/kg vitamin K1 (Konakione, Roche Pharmaceuticals). The warfarin/vitamin K1 treatment started on postnatal day 1 and continued until the animals were killed by carbon dioxide inhalation after 5 weeks (3 rats), 7 weeks (2 rats) or 12 weeks (4 rats). There was also one rat treated for the first 12 weeks postnatal weeks and killed at 1 years. There were five untreated control rats killed at 7 weeks (2 rats), 12 weeks (2 rats) and 1 years (1 rat).

The vascular system and various organs were dissected out and stained for calcium using the alizarin red technique (Kimmel & Trammel 1981). Some tissue was fixed in glutaraldehyde in phosphate buffer and embedded in Spurr's plastic and sectioned at 1 micron. Sections were stained for calcium using von Kossa's silver technique (Drury & Wallington 1976) and counterstained with 0.25% azure II in 0.5% sodium borate.

Results

In all of the warfarin-treated rats there was calcification along the abdominal aorta and its branches and the pulmonary trunk and its branches as demonstrated by alizarin red-staining (Figure 1a,d-g). Veins were not affected. Calcification was never complete but consisted of numerous centres of varying size. Some vessels showed a circumferential arrangement of calcification (Figure 1a). The extent and distribution of the arterial calcification varied between animals but appeared to be unrelated to duration of treatment.

In some rats the aorta showed relatively little calcification compared with that seen in the branches of the aorta (Figure 1d). In one rat the aorta showed only a thin longitudinal strip of calcification. The rat treated for 12 weeks and allowed to recover for 9 months had widespread calcification in the aorta and its branches (Figure 1a) suggesting that there is no regression of the calcification after cessation of treatment. The coronary arteries were frequently affected (Figure 1d) but it was not possible to see if the pulmonary and aortic valves were involved. The most heavily calcified vessels were usually the pulmonary arteries (Figure 1d,e).

In all treated animals the renal arteries were heavily calcified and they could be seen extending into the tissue of the kidney (Figure 1g). One kidney showed dense staining in the renal calices with particulate matter extending into the ureter (Figure 1f). These were presumably small renal stones. Unfortunately the other kidney was not retained. This animal did not appear to be sick prior to euthanasia. Two other rats showed internal renal staining that was radially orientated.

Von Kossa-stained sections revealed that the

Figure 1 This figure shows blood vessels from rats treated with warfarin and vitamin K from birth. (b) and (c) are sections stained for calcium with von Kossa's stain and (a) and (d -g) are from whole mounts stained for calcium with alizarin red. a. Common iliac artery from a rat treated for 12 weeks from birth and killed at 1 year. The red staining corresponds to calcified areas with some circumferential arrangement. b. A section through the wall of the aorta at the level of the aortic valves (v) from a rat treated for 12 weeks. The two black areas are calcification in the aortic wall. The valve does not show any calcification. c. An enlargement of the lower area of calcification seen in 1b. d. Heart and great vessels from a rat treated for 12 weeks from birth. There is heavy staining in the pulmonary arteries (p) and coronary artery (c). The aorta (a) and its branches show less calcification. e. Heart, lungs, vessels and trachea from a rat treated for 5 weeks. The pulmonary arteries (p) extending from the lungs are heavily stained. f. Kidney and adjacent aorta and vessels from a rat treated for 12 weeks. The kidney contains a large mass of alizarin-stained material apparently in the collecting system. The material extends into the ureter (u) and appears granulated. The aorta and its branches including the renal arteries show considerable staining. g. Kidney and adjacent aorta and vessels from a rat treated for 12 weeks from birth. The aorta and its branches show considerable staining. The stained renal arteries can be seen extending into the kidney.

calcification originated at multiple sites along the vessel walls located in the elastic lamellae of the media (Figure 1b,c). At some sites of calcification the vessel wall appeared to be thin and did not show the folding associated with the elastic tissue of the vessel wall normally seen in histological sections. Cerebral arteries and veins and capillaries did not appear to be affected. There was no evidence of calcification in any of the control tissues stained with alizarin or von Kossa's technique.

Discussion

Rationale for the animal model

The animal model used in this study is not completely analogous to the human receiving warfarin. Ideally we would have treated the rats with warfarin on its own to cause an increased clotting time as occurs in the human. However, it is difficult to maintain rats on a therapeutic dose of warfarin due to coprophagia which results in unpredictable dosing. There is also the risk of bleeding associated with blood sampling to monitor clotting time. Instead we used a treatment protocol first described by Price and Williamson (1981). Concomitant administration of warfarin and vitamin K1 to rats results in normal formation of blood clotting factors. This occurs because the liver can utilize the supplemental vitamin K to overcome the blockage to vitamin K recycling caused by the warfarin. Extrahepatic sites appear to be unable to utilize the excess vitamin K1 (Price & Kaneda 1987), so the extrahepatic vitamin K-dependent proteins such as matrix gla protein and osteocalcin are likely to be noncarboxylated and nonfunctional.

Pathology of the treated rats

The calcification seen in the arterial system of the warfarin treated rats appears to be basically the same as described in the MGP knock-out mice (Luo *et al.* 1997). There was no obvious increase in the extent of calcification between 7 and 12 weeks treatment but comparison was difficult as there was considerable variation in the extent and distribution of arterial calcification between animals having the same duration of treatment. In a study using the same warfarin/vitamin K treatment regimen in rats (Price *et al.* 1998) it was reported that there was no calcification after one week of treatment, calcification started to appear after 2 weeks and continued to increase up to 5 weeks which was the duration of the study.

This is strong evidence that the calcification seen in the rats is a consequence of the formation of non- or poorly functional MGP. In mice at least, MGP is highly expressed in the smooth muscle cells of the media of the arteries but is not expressed in other nonvascular muscle cells or in the myocardium (Luo *et al.* 1997).

Osteocalcin is unlikely to be involved in the pathology as it is only expressed by osteoblasts and in osteocalcin knockout mice there was no reported pathology of the blood vessels (Ducy *et al.* 1996). However, it has been reported that osteocalcin is expressed in calcified arteries (Bostrum *et al.* 1995).

It has been speculated that MGP normally binds excess calcium in the vessel wall and the calcium/MGP complex is exported into the circulation (Proudfoot *et al.* 1998). In this way MGP would prevent hydroxyapatite formation. If the MGP was absent, as in the knockout mice, or noncarboxylated as is likely in the present study, then hydroxyapatite crystals might appear in the vessel wall and lead to progressive calcification. The calcification in the MGP knockout mice was identified as apatite (Luo *et al.* 1997). MGP may be part of a complex of proteins in the wall of arteries that prevent calcification. Another protein, osteoprotegerin (OPG), is also localized in the smooth muscle layer (media) of aortic and renal arteries and OPG deficient mice also show calcification of these arteries (Bucay *et al.* 1998).

Although the calcification in the rats appeared to be severe and there was vessel wall thinning there were no premature deaths as seen in the MGP knockout mice. In the mice the calcification was a continuous sheet encompassing the arterial media and most of the animals died of blood vessel rupture in the first 8 weeks of life. This difference in severity may be related to incomplete inhibition of MGP carboxylation by the warfarin treatment in rats (Price *et al.* 1998).

In the other rat study using the same warfarin/vitamin K treatment regimen (Price *et al.* 1998) it was shown that 4-weeks treatment resulted in a 4-fold increase in calcium and a 13-fold increase in MGP in the aorta while serum MGP was decreased 3-fold. A suggested explanation is that when noncarboxylated MGP is formed it can still bind to some matrix factor in the blood vessel wall but it cannot bind calcium. As calcium levels start to increase in the vessel wall more MGP is produced but it cannot sequester the calcium. Hydroxyapatite crystals form with a chain reaction resulting in focal calcification. If MGP is only released from the vessel wall when it has bound calcium (Proudfoot *et al.* 1998) this would explain the reduced serum MGP levels.

Kidney calcification

The severe calcification seen in one kidney in this study is in agreement with the markedly increased calcium

levels in the kidney measured by Price *et al*. (1998). Although MGP has been detected in the kidney (Fraser & Price 1988) it may be associated with blood vessels rather than the kidney tissue. There is evidence of another vitamin K-dependent protein in the kidney, nephrocalcin. This protein has two or three gla residues and was initially identified in human urine. It was shown to inhibit the formation and growth of calcium oxalate crystals which form some renal stones (Nakagawa *et al*. 1983). The related carboxylase is located in the renal tubule cells (Friedman *et al*. 1982) and it has been suggested that its product, presumably carboxylated nephrocalcin, is secreted into the urine.

There have been isolated reports of renal stones associated with warfarin therapy in humans (McLain *et al*. 1980; Miller *et al*. 1991; Mikami *et al*. 1992). It is also known that haematuria is associated with warfarin therapy and it has been suggested that this is not due to the anticoagulant effect of warfarin but is secondary to microscopic calcium oxalate stones irritating or ulcerating the renal collecting system (Fowler 1986).

Evidence that warfarin affects MGP and osteocalcin in humans

In humans, the possible effects of warfarin on MGP does not appear to have been studied. In contrast there are a number of studies showing that warfarin treatment in humans affects the carboxylation state of osteocalcin. Osteocalcin is secreted by osteoblasts and active carboxylated osteocalcin binds strongly to hydroxyapatite in bone. Serum osteocalcin levels are thought to reflect bone formation (Price & Williamson 1981). Studies in patients receiving warfarin consistently show increased circulating levels of under-carboxylated osteocalcin (Menon *et al*. 1987; Pietschmann *et al*. 1988; van Haarlem *et al*. 1988; Merle & Delmas. 1990; Plantalech *et al*. 1991; Jie *et al*. 1993). In one study where subjects were given a minidose (1 mg/day) of warfarin for 7 days the under-carboxylated osteocalcin in serum increased 170% compared to baseline (Sokoll *et al*. 1995). In other studies with long-term anticoagulated patients circulating normal osteocalcin was about 25–40% of control values (Menon *et al*. 1987; van Haarlem *et al*. 1988).

Hence by comparison with the effects of warfarin on osteocalcin, it is reasonable to suppose that humans on long-term warfarin therapy would have under-carboxylated MGP and hence would be at risk of arterial calcification. An association between warfarin therapy and arterial calcification in humans has not been reported. Premature calcification of laryngeal structures has been reported in children receiving long-term warfarin therapy (Rifkin & Pritzker 1984; Taybi & Capitanio 1990) with calcification related to duration of treatment (Moncada *et al*. 1992). This group may be appropriate to examine for signs of arterial calcification uncomplicated by the ageing process.

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